REMARKS

Reconsideration of the present application in view of the above amendments and the following remarks is respectfully requested. Claims 435, 436, 438, 439, 446 and 447 are pending. Claims 438 and 439 have been amended to facilitate allowance and without acquiescing to the rejections in the Office Action. More specifically, subsection (i) of claim 438 has been amended to simplify the definition of the scFv binding domain polypeptide, which now refers to only one sequence reference number and reads as follows: "amino acids 23-265 of SEQ ID NO:246." Because the sequence of amino acids 23-265 of SEQ ID NO:246 is identical to the sequences recited in subsection (i) of previously pending claim 438, no new matter has been added. In addition, subsection (ii) of claim 438 has been amended to accurately identify the hinge sequence, which spans amino acids 269-283 of SEQ ID NO:246. Support for this amendment to subsection (ii) of claim 438 may be found, for example, in the last paragraph on page 113 of the substitute specification submitted February 26, 2009, which defines wild type immunoglobulin hinge peptides. Based on this definition, a wild type human IgG1 hinge peptide has a sequence of EPKSCDKTHTCPPCP. The IgG1 hinge peptide of SEQ ID NO:246 corresponds to amino acids 269-283 and is a mutated version of a wild type hinge with a sequence of EPKSCDKTHT**S**PPC**S** (wherein the two underlined and bold "S" letters indicate a serine substitution for cysteine and a serine substitution for proline, respectively, of the wild type hinge). Claim 439 has been amended to correct a typographical error.

As an initial matter, Applicants wish to thank Examiner Bristol for discussing the present application with Applicants' representatives during an in-person interview on December 8, 2009. During the interview, Examiner Bristol and Applicants' representatives discussed the obviousness rejections in the Office Action. More specifically, Applicants' representatives discussed the references cited in the Office Action, noting certain deficiencies in their disclosures. In addition, Applicants' representatives proposed amendments to claim 438, as submitted above, to clarify the claimed subject matter.

REJECTION UNDER 35 U.S.C. § 103(a)

Claims 435, 436, 438, 439, 446 and 447 stand rejected under 35 U.S.C. §103(a) as obvious over Robinson *et al.* (WO 88/04936, "Robinson") in view of Welschof *et al.* (Human

Immunol. 60:282-90, 1999, "Welschof") and Schilling (US 2005/0084933, "Schilling"). More specifically, it is asserted in the Office Action that (1) Robinson discloses a chimeric mouse 2H7 antibody where the mouse immunoglobulin constant domains are replaced with the human IgG1 constant domains including the human IgG1 hinge, CH1, CH2 and CH3 domains, and where the human constant domains confer ADCC and CDC activities while retaining antigen specificity; (2) Welschof discloses scFv antibodies and teaches that mutations in the hinge help prevent auto-antibody responses to a recombinant antibody administered in vivo where the auto-antibody recognizes epitopes in the hinge domain; and (3) Schilling teaches (a) generating a CTLA4 immunoglobulin fusion protein that comprises the extracellular domain of CTLA4 joined to an immunoglobulin constant region, which includes a hinge that can have changes in any or all of the cysteines, a CH2 region that can have a mutation in a proline, and a CH3 region.

Applicants respectfully traverse this ground of rejection. Before presenting any arguments, Applicants respectfully submit that any discussion provided herein does not address the comments made in the Office Action on page 7, last paragraph through page 8. The references named on these pages, "Ledbetter" and "Inouye," are not of record, and replacing "Ledbetter" and "Inouye" with "Robinson" and "Welschof," respectively, as suggested by the Examiner via phone on December 4, 2009, does not fully clarify this portion of the rejection. Nonetheless, the rest of the rejection will be addressed herein. Applicants submit that the cited references of Robinson, Welschof and Schilling, either taken alone or in combination, fail to teach or suggest the fusion protein claimed in the present application as discussed during the interview and further in detail below.

First, Applicants submit that the cited references, alone or in combination, fail to teach or suggest a proline to serine mutation in the hinge region. More specifically, Robinson discloses a chimeric anti-CD20 antibody that is an anti-human CD20 binding domain of mouse monoclonal antibody 2H7 incorporated into a human IgG1 antibody scaffold. As acknowledged in the Office Action, Robinson fails to teach or suggest a mutated core hinge region as recited in the currently pending claims.

Welschof fails to remedy this deficiency of Robinson. Welschof describes that naturally arising anti-$F(ab')_2$ auto-antibodies recognize an antibody hinge sequence and, therefore, this epitope was mapped (*see* Abstract and left column at page 283). Welschof probed

mutated double chain hinge peptide fragments fixed on cellulose membranes (*see* Abstract) and found that the middle hinge region cysteines and prolines are part of the anti-F(ab')$_2$ epitope (*see* right column at page 285, abstract, and Figure 3). But, Welschof neither taught nor suggested such mutated hinge peptides for use in antibodies, much less in a fusion protein as claimed. In other words, the peptides on the cellulose membranes were merely a tool to map an antibody epitope and nothing more. A person of skill in the art at the time of the present invention would not have had a reasonable expectation of success in using such mutated hinge fragments since their function properties were unknown, particularly with mutations in the highly conserved prolines of the IgG1 core sequence. Moreover, the IgG1 hinge fragments used by Welschof had only three amino acids of the 10 amino acid IgG1 upper hinge region (*i.e.*, only THT from EPSCDKTHT; *see* Figure 3) along with the core and lower hinge sequences. These truncated hinges would not have provided one of ordinary skill in the art motivation to use these IgG1 hinge fragments in a fusion protein as claimed because the length of an upper hinge region was known to correlate with the segmental flexibility of an antibody and, thus, it would have been unpredictable whether such hinge fragments would provide the proper functional structure for the fusion proteins (*see*, page 2, lines 26-30 of substitute specification submitted February 26, 2009, citing Shin et al. (1992) *Immunol. Rev.* 130:87).

Moreover, even assuming for the sake of argument that one of ordinary skill in the art were to modify the human IgG1 hinge region in view of Welschof, such a change would not be desirable because Welschof indicates that the anti-F(ab')$_2$ autoantibodies suppress the deleterious effects of autoreactive B cells (*see*, right column at page 282) and thus are beneficial. In addition, Welschof further indicates that anti-F(ab')$_2$ autoantibodies have considerable potential therapeutic value (*see*, the first sentence in the second full paragraph of the left column on page 283). Accordingly, if as asserted in the Office Action, administering a fusion protein that comprises a human IgG1 hinge would induce the production of anti-F(ab')$_2$ autoantibodies, one of ordinary skill in the art would not have been motivated to mutate the IgG1 core hinge region to reduce the production of the anti-F(ab')$_2$ autoantibodies because such changes would negate the beneficial effects of these autoantibodies.

Schilling also fails to teach or suggest a proline to serine mutation in the hinge region. Although Schilling states that the immunoglobulin moiety may comprise mutations, for

example, substitutions in any or all of the cysteine residues within the hinge domain, it does not teach or suggest any proline to serine substitution in the hinge region (*see* paragraph [0212]]. The fusion protein shown in Figure 8 contains cysteine to serine substitutions at positions 130, 136 and 139 and a proline to serine substitution at position 148. The three cysteine to serine substitutions are in the hinge region, whereas the proline to serine substitution is in the CH2 region, not in the hinge region.

Second, Applicants submit that the cited references, alone or in combination, fail to teach or suggest a leucine to serine substitution at position 11 of the heavy chain variable regions as in SEQ ID NOS:372, 246, 370, 268 and 276 recited in the claims of the present application. None of the cited references mention or suggest the above-noted substitution in a heavy chain variable region.

Third, Applicants further submit that the cited references, taken alone or in combination, fail to teach or suggest a fusion protein that comprises an scFv binding domain polypeptide, a mutant hinge region polypeptide, a CH2 region polypeptide, and a CH3 region polypeptide as claimed in the present application. More specifically, Robinson teaches a chimeric anti-CD20 antibody in which the mouse immunoglobulin constant domains are replaced with the human IgG1 constant domains including the human IgG1 hinge, CH1, CH2, and CH3 domains. Welschof used the pHOG21 vector to generate naked scFv molecules to probe the hinge peptide fragments (*see* paragraph with subtitle "Isolation and Expression of anti-$F(ab')_2$ scFv Antibodies" in the left column on page 284). It was known in the art that the pHOG21 vector encodes only scFv molecules, referred to by Welschof as "scFv antibodies," which includes only the heavy and light chain variable domains linked together (*see* Peter *et al.*, Circulation 101:1158-64, 2000, in particular Figure 2 on page 1159, enclosed) – that is, the scFv antibodies of Welschof do not contain hinge or Fc region polypeptides as presently claimed. Thus, combining Robinson and Welschof as indicated in the Office Action would arrive at a CD20-specific scFv polypeptide, rather than a CD20-specific scFv-Ig fusion protein as claimed in the present application. In addition, Schilling relates to a fusion protein that comprises the extracellular domain of CTLA4 and does not teach or suggest the substitution of the extracellular domain of CTLA4 with an scFv polypeptide, especially a CD20-specific scFv polypeptide.

Thus, combining Schilling with Robinson and Welschof would not have arrived at the fusion protein as claimed in the present application.

In view of the above remarks, Applicants submit that this ground of rejection under 35 U.S.C §103(a) has been overcome. Withdrawal of this rejection is respectfully requested.

The Director is authorized to charge any additional fees due by way of this Amendment, or credit any overpayment, to our Deposit Account No. 19-1090.

Applicants believe that the remaining claims of the present application are now allowable. Favorable consideration and a Notice of Allowance are earnestly solicited.

Respectfully submitted,

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Enclosure:
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Construction and Functional Evaluation of a Single-Chain Antibody Fusion Protein With Fibrin Targeting and Thrombin Inhibition After Activation by Factor Xa

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Background—Recombinant technology was used to produce a new anticoagulant that is preferentially localized and active at the site of the clot.

Methods and Results—The variable regions of the heavy and light chains of a fibrin-specific antibody were amplified by polymerase chain reaction (PCR) with hybridoma cDNA. To obtain a functional single-chain antibody (scFv), a linker region consisting of $(Gly_4Ser)_3$ was introduced by overlap PCR. After the scFv clones were ligated with DNA encoding the pIII protein of the M13 phage, high-affinity clones were selected by 10 rounds of panning on the Bβ15-22 peptide of fibrin (β-peptide). Hirudin was genetically fused to the C-terminus of the variable region of the light chain. To release the functionally essential N-terminus of hirudin at the site of a blood clot, a factor Xa recognition site was introduced between $scFv_{59D8}$ and hirudin. The fusion protein was characterized by its size on SDS-PAGE (36 kDa), by Western blotting, by its cleavage into a 29-kDa (single chain alone) and 7-kDa (hirudin) fragment, by its binding to β-peptide, and by thrombin inhibition after Xa cleavage. Finally, the fusion protein inhibited appositional growth of whole blood clots in vitro more efficiently than native hirudin.

Conclusions—A fusion protein was constructed that binds to a fibrin-specific epitope and inhibits thrombin after its activation by factor Xa. This recombinant anticoagulant effectively inhibits appositional clot growth in vitro. Its efficient and fast production at low cost should facilitate a large-scale evaluation to determine whether an effective localized antithrombin activity can be achieved without systemic bleeding complications. **(*Circulation.* 2000;101:1158-1164.)**

Key Words: anticoagulants ■ antibodies ■ thrombosis ■ molecular biology

Inhibition of thrombin by either the indirect thrombin inhibitor heparin or direct thrombin inhibitors such as hirudin reduces thrombus formation after arterial injury in animal models[1] and in humans with unstable coronary syndromes.[2,3] Furthermore, thrombin inhibitors potentiate fibrinolysis induced by plasminogen activators.[4] Several animal experiments demonstrated that hirudin is more effective than heparin in preventing platelet-dependent arterial thrombosis, rethrombosis after reperfusion, and thrombus growth.[4–7] However, clinical trials with direct thrombin inhibitors have only been partially successful.[8] High concentrations of hirudin were very effective in inhibiting thrombin but are associated with frequent hemorrhagic complications.[4] A strategy for circumventing this problem is the targeting of hirudin to fibrin.

Fibrin targeting can be achieved with the monoclonal antibody (mAb) 59D8, which selectively binds to the amino-terminus of the fibrin β-chain that becomes exposed after cleavage of fibrinopeptide B by thrombin.[9] Because exposure of this epitope is an early event in the conversion of fibrinogen to fibrin, it is likely that mAb 59D8 accumulates at sites of high thrombin activity, such as a developing arterial clot.[9] Coupling of mAb 59D8 to plasminogen activators resulted in enhanced thrombolytic potency and specificity in vitro and in vivo.[10,11] A chemical conjugate between hirudin and 59D8 effectively inhibited fibrin deposition on experimental clots[12] and demonstrated potent antithrombotic activity in nonhuman primates.[13] Nevertheless, chemical coupling of hirudin to mAbs has several limitations, the major ones being low yield and loss of hirudin activity.[12] We tried to bypass these limitations by the use of recombinant technology.

Because hirudin needs a free amino- as well as a free carboxy-terminus for antithrombin activity,[14] a direct fusion

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Presented in part at the annual meeting of the American College of Cardiology, New Orleans, La, March 7–10, 1999, and published in abstract form (*J Am Coll Cardiol.* 1999;33:2A).

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at the termini of hirudin was expected to result in a functional loss. Therefore, a factor Xa cleavage site was introduced between mAb 59D8 and hirudin. This cleavage site was chosen for 2 reasons. First, factor Xa cleaves at the C-terminus of its recognition sequence (Ile-Glu-Gly-Arg) and thus liberates the free amino-terminus of hirudin. Second, factor Xa is a major part of the activated coagulation system at the site of arterial clots[15] and may therefore allow a preferential liberation of functional hirudin at the clot. Without an activated coagulation system, the fusion protein would be inert. However, as a clot develops, the combination of fibrin targeting and dependence on cleavage by factor Xa could result in an effective thrombin inhibition at the clot without systemic anticoagulation.

Methods

Materials and Cells

Horseradish peroxidase (HRP)–conjugated sheep anti-M13 mAb and mouse anti-E-tag mAb were obtained from Pharmacia, mouse anti-c-myc mAb from Cambridge Research Biochemicals, and goat anti-mouse HRP-conjugated polyclonal antibody (Ab) from Dianova. Bβ15-22, also termed β-peptide, with the amino acid sequence Gly-His-Arg-Pro-Leu-Asp-Lys, was purchased from MWG Biotech. Hirudin was a gift of Knoll AG (Ludwigshafen, Germany). The hybridoma secreting the fibrin-specific mAb 59D8 was generated as described previously,[9] and cells were grown on DMEM, 10% fetal calf serum, 2 mmol/L L-glutamine, penicillin (10 IU/mL), and streptomycin (10 μg/mL) (all from Gibco) with 5% CO_2 at 37°C.

Construction of a Functional Single-Chain Antibody

cDNA of 59D8 hybridoma cells was prepared with mRNA purification columns (oligo-dT) and M-MuLV (both from Pharmacia). Amplification of the antibody variable regions and the insertion of the linker sequence were achieved by polymerase chain reaction (PCR). Primer mixes that contained sequences from conserved regions of the variable regions of the heavy (V_H) and light (V_L) chains were obtained from Pharmacia. The linker sequence $(Gly_4Ser)_3$ was inserted by the addition of a linker fragment.

Clone Selection With the M13 Phage System

The PCR products encoding the functional single-chain antibody fragments (scFv) were cloned into the vector pCANTAB5E (Pharmacia). In this vector, an amber stop codon allows expression of soluble scFv in the nonsuppressor *Escherichia coli* strain HB2151 and display of scFv on the M13 phage surface by fusion to the pIII adsorption protein in the suppressor strain TG1. The supernatant of TG1 clones was used for the following panning procedure: A tissue culture flask with a surface area of 25 cm^2 was coated with 50 μg of β-peptide at 4°C overnight, washed 5 times with PBS, and blocked for 2 hours at 37°C with 2% nonfat dry milk in PBS. The phage-containing supernatant was added and incubated for 2 hours at 37°C. Nonadhering phages were removed by washing 20 times with PBS. A TG1 culture was added to the flask for reinfection with bound phages and incubated for 1 hour at 37°C at 250 rpm. This panning procedure was repeated 9 times. Positive clones were tested for phage binding on immobilized β-peptide by use of an HRP-conjugated anti-M13 sheep mAb. The best binding clones with the expected fragment size (≈750 bp) were used to transform HB2151. Periplasmic extracts from the individual clones were analyzed for binding to immobilized β-peptide by an anti-E-tag mAb.

Cloning of $scFv_{59D8}$ Into the Expression Vector pHOG21, Fusion With the Factor Xa Recognition and Hirudin Sequences, and Transfer to pOPE51

DNA of scFv clone 33 was cloned into pHOG21[16], mutated at position 6 to glutamine,[17] and cloned into pOPE51[18] (Figure 2).

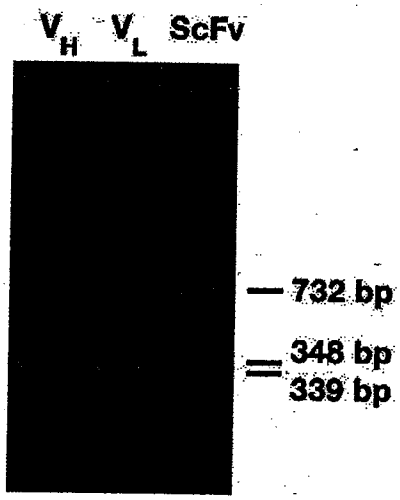

Figure 1. PCR products for V_H, V_L, and scFv. Electrophoresis was performed on 1% agarose gel, and PCR products were visualized by ethidium bromide.

DNA coding for hirudin (Biermann) was used as a template for PCR with the sense primer CAGCAAGATCTAAACTCAAGCGGC-**ATCGAAGGTCGT**GTTGT-TTACACCGACTGTACTG and the antisense primer AGATGATCTAGAGGATCCTTACTGCAGAT-ATTCTTCTGGG. The factor Xa recognition sequence (bold) and the restriction site *BglII* (underlined) are encoded by the sense primer and the restriction site *XbaI* (underlined) by the antisense primer. The ligation products were transformed into XL1-blue.

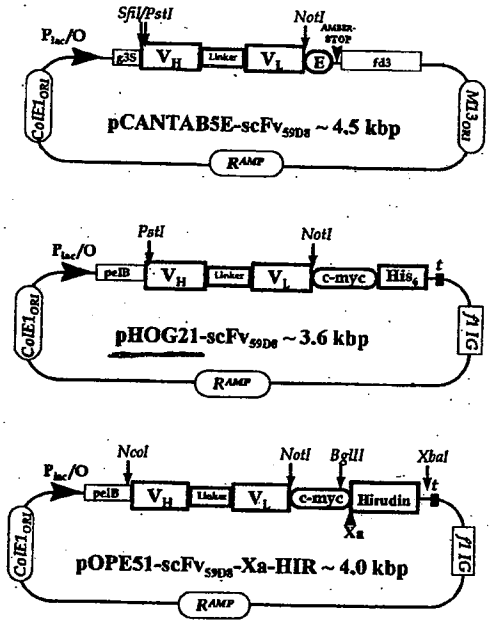

Figure 2. Maps of pCANTAB5E-$scFv_{59D8}$, pHOG21-$scFv_{59D8}$, and pOPE51-$scFv_{59D8}$-Xa-HIR. R^{AMP} indicates ampicillin resistance gene; $ColE1_{ORI}$, origin of replication of *E coli*; $M13_{ORI}$, origin of replication of filamentous phage M13; *f1 IG*, filamentous intergenic region; P_{lac}/O, lactose regulatable promoter/operator; *g3S*, signal sequence of pIII; *fd3*, coding region of pIII gene of M13; *pelB*, leader peptide sequence of pectate lyases pelB; E, E tag with amino acid sequence GAPVPYPDPLEPR, *c-myc*, c-myc tag with amino acid sequence EQKLISEEDLN; His_6, repeat of 6 histidines; *Xa*, factor Xa recognition site; and *AMBER-STOP*, amber stop codon TAG.

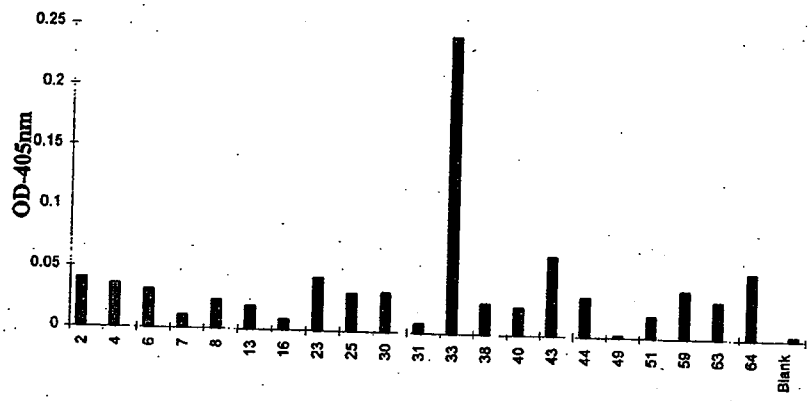

Figure 3. Binding of $scFv_{59D8}$ clones to immobilized β-peptide. After immobilization of β-peptide on 96 well plates, wells were washed and incubated with different scFv clones. Unbound scFvs were washed away, and bound scFvs were detected by an anti-E-tag mAb and an HRP-labeled goat anti-mouse Ab.

Preparation of scFv From Inclusion Bodies

From overnight cultures of XL1-blue, 250 μL was transferred to 5 mL of LB medium containing 100 μg/mL ampicillin and 100 mmol/L glucose and incubated at 37°C and 280 rpm until an $OD_{600\,nm}$ of 0.8 was reached. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG; 20 μmol/L) and cultured at room temperature for 4 hours. Cells were then centrifuged (6000 rpm, 15 minutes) and resuspended in 165 μL of ice-cold buffer (50 mmol/L Tris-HCl, 100 mmol/L NaCl, 1 mmol/L EDTA, pH 7.0). After freezing and thawing, the sample was centrifuged (12 000*g*, 4°C, 30 minutes), resuspended in 500 μL of ice-cold TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.4), and incubated for 1 hour at room temperature. Lysozyme (Boehringer Mannheim) was then added to a final concentration of 200 μg/mL, and the incubation was continued for 1 hour, followed by the addition of NaCl (0.5 mol/L) and Triton-X-100 (2.5%) and a final incubation for 1 hour. After centrifugation (12 000*g*, 4°C, 1 hour), the pellet was washed twice with 3 mol/L urea, and 50 mmol/L Tris-HCl (pH 7.0) and finally solubilized by rotation overnight at 4°C in 250 μL of 6 mol/L GdHCl, 100 mmol/L Tris-HCl, pH 7.0. After centrifugation (12 000*g*, 4°C, 1 hour), the supernatant was dialyzed against TA buffer (0.4 mol/L arginine-HCl in 100 mmol/L Tris-HCl, pH 7.0).

ELISA With Immobilized β-Peptide

Microtiter plates were coated with 1 μg of β-peptide or the control peptide GRGDSP in 100 μL of 0.05 mol/L Na_2CO_3 (pH 9.6) overnight at 4°C. The plate was then washed 4 times with PBS and blocked with blocking buffer (2% nonfat dry milk in PBS) for 2 hours at room temperature. Samples (100 μL) were incubated for 2 hours at room temperature. After the plate was washed 5 times with PBS, 100 μL of mAb solution (either anti-M13 mAb, anti-c-myc mAb [both 1 to 5000 diluted], or 1 μg/mL anti-E-tag mAb) in blocking buffer was added and incubated for 2 hours at room temperature. Either ABTS (Sigma) or TMB solution (Biorad) was used, and samples were measured at wavelengths of 405 or 655 nm, respectively.

Purification by Immobilized Metal Affinity Chromatography and Ionic Exchange Chromatography of $scFv_{59D8}$ Expressed in the pHOG21 Vector

A 10-mL column of chelating sepharose (Pharmacia) was equilibrated with 0.1 mol/L $CuSO_4$. The samples were loaded in 50 mmol/L Tris-HCl, 1 mol/L NaCl, pH 7.0. After the column was washed with 200 mL of 50 mmol/L Tris-HCl, 1 mol/L NaCl, 50 mmol/L imidazole, pH 7.0, the bound scFv construct was eluted with 40 mL of 50 mmol/L Tris-HCl, 1 mol/L NaCl, 250 mmol/L imidazole, pH 7.0. The eluate was dialyzed against PBS buffer and further purified by ionic exchange chromatography on a MonoS column (Pharmacia) in 50 mmol/L MES buffer (pH 6.5) with a 0 to 1 mol/L NaCl gradient.

Affinity Chromatography of scFv and Factor Xa Cleavage

Coupling of β-peptide to sepharose was performed as described previously.[10] Columns containing β-peptide–conjugated sepharose were loaded and washed with TA buffer. Bound protein was eluted by 0.1 mol/L glycine, pH 2.8, and 1-mL fractions were collected and adjusted to pH 7.0 with 0.5 mol/L Tris buffer. Fractions containing significant amounts of product were pooled and dialyzed against TA buffer. For factor Xa cleavage, typically 150 μg of $scFv_{59D8}$-Xa-hirudin (2 μg/μL) was cleaved by 15 μg of factor Xa (1 μg/μL, Boehringer Mannheim) for various times in 50 mmol/L Tris-HCl, 100 mmol/L NaCl, and 1 mmol/L $CaCl_2$ (pH 8.0) at room temperature.

Measurement of Thrombin Inhibition by $scFv_{59D8}$-Xa-Hirudin After Factor Xa Cleavage

Inhibition of thrombin was determined by cleavage of the chromogenic substrate S-2238 (Chromogenix). After factor Xa (0.1 μg/μL) cleavage (5 hours, room temperature), 20 μL of thrombin solution (human thrombin, 2.5 U/mL; Sigma) was added to 100 μL of sample in assay buffer (20 mmol/L sodium dihydrogen carbonate, 0.15 mol/L NaCl, and 0.1% bovine serum albumin, pH 7.4) and incubated at room temperature for 10 minutes. S-2238 (50 μL, 0.833 mg/mL) was then added, and after 10 minutes of incubation, the reaction was stopped by the addition of 50 μL of 20% acetic acid. Absorbance was measured at 405 nm.

Whole Blood Clot Assay

Except for minor modifications, clot assays were performed as described previously.[12] Clots were initiated by the addition of $CaCl_2$ (16.6 mmol/L) and 2.5 vol% of Actin[7] FS-activated PTT reagent (Dade International) to anticoagulated blood (citric acid, 11 mmol/L) from healthy volunteers. Whole blood was immediately drawn into a silicone tubing (4-mm inner diameter), and clots were allowed to form at 37°C for 1 hour. Quantification of clot size was performed by labeling of blood with ^{125}I-fibrinogen (Amersham) to a final activity of 37 500 cpm/mL. The silicon tubing was cut into 1.5-cm fragments, and the formed clots were extruded and washed 5 times in TA buffer. In each assay, the starting size of clots chosen for further experiments varied not more than ±5%. Appositional clot growth was evaluated by the incubation of clots for 10 hours at 37°C on a rotator (60 rpm). Clots were incubated in recalcified citrated whole blood (trace labeled with ^{125}I-fibrinogen at a final activity of 112 500 cpm/clot) either with the addition of native hirudin or $scFv_{59D8}$-Xa-hirudin or without addition. The clots were then washed 10 times with TA buffer, and inhibition of appositional clot growth was evaluated on a γ-counter.

Results

To construct a single-chain antibody directed against fibrin (scFv), mRNA was prepared from 5×10^7 59D8 hybridoma cells and reverse transcribed with an oligo-dT primer. The

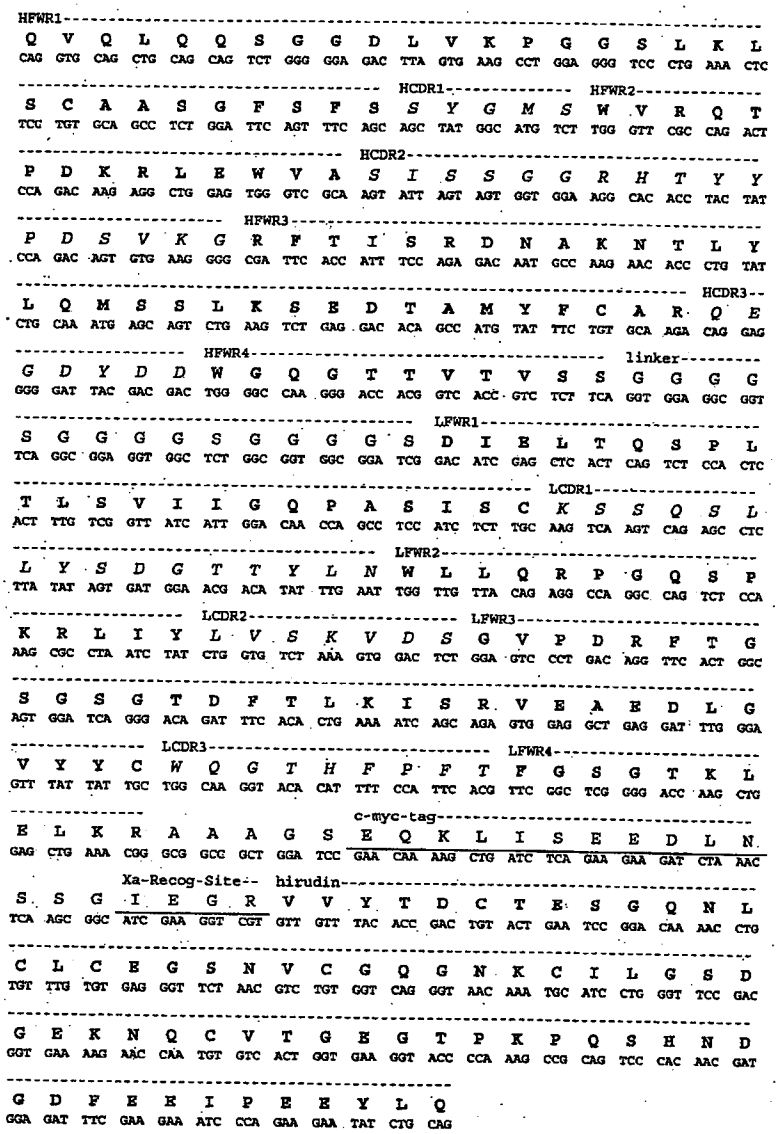

Figure 4. Protein and DNA sequence of $scFv_{59D8}$-Xa-hirudin. The hirudin sequence and partially conserved frameworks are in boldface type (HFWRs indicates frameworks of heavy chain; LFWRs, frameworks of light chain), and CDRs (complementarity determining regions) are in italic type. c-myc-tag and factor Xa recognition sequence are underlined.

variable regions of the heavy and light chains (V_H and V_L, respectively) were amplified by polymerase chain reaction (PCR) with primers that anneal to conserved regions at the 5′- and 3′-ends of the variable regions. PCR products of 348 and 339 bp were obtained for V_H and V_L, respectively (Figure 1). After addition of a $(Gly_4Ser)_3$ linker by fusion PCR, the scFv product (Figure 1) was cloned into pCANTAB5E (Figure 2) for phage display of the scFv clones. After 10 rounds of panning on immobilized β-peptide, 144 phage clones were tested for binding to the β-peptide by phage ELISA with a horseradish peroxidase (HRP)–conjugated mAb against M13 and by DNA restriction analysis. Twenty-four clones with strong binding to β-peptide and the expected size of the insert were used for transformation of HB2151. The binding properties of soluble scFvs secreted into the supernatant were compared by ELISA with a mouse anti-E-tag mAb and a secondary HRP-conjugated goat anti-mouse antibody (Ab) (Figure 3). Clone 33 demonstrated the best binding properties (Figure 3) and was therefore chosen for sequencing and further characterization. Complementarity determining regions and framework regions of both variable regions and the linker region are highlighted in Figure 4.

For enhanced expression and purification of soluble $scFv_{59D8}$, clone 33 was transferred to the expression vector pHOG21 (Figure 2). This plasmid contains a tag sequence coding for 6 histidine residues at the scFv C-terminus, thus facilitating purification by immobilized metal affinity chromatography. However, an additional purification step with ion-exchange chromatography was necessary to obtain a pure product (Figure 5A). An analysis of several eluted fractions by SDS-PAGE is shown in Figure 5B. The functional integrity of the highly purified $scFv_{59D8}$ was tested by binding on immobilized β-peptide (Figure 5C). The yield of purified $scFv_{59D8}$ was 0.2 mg from 1 L of bacterial culture.

To further increase the yield, glutamic acid at position 6 of the heavy chain was mutated by PCR to glutamine,

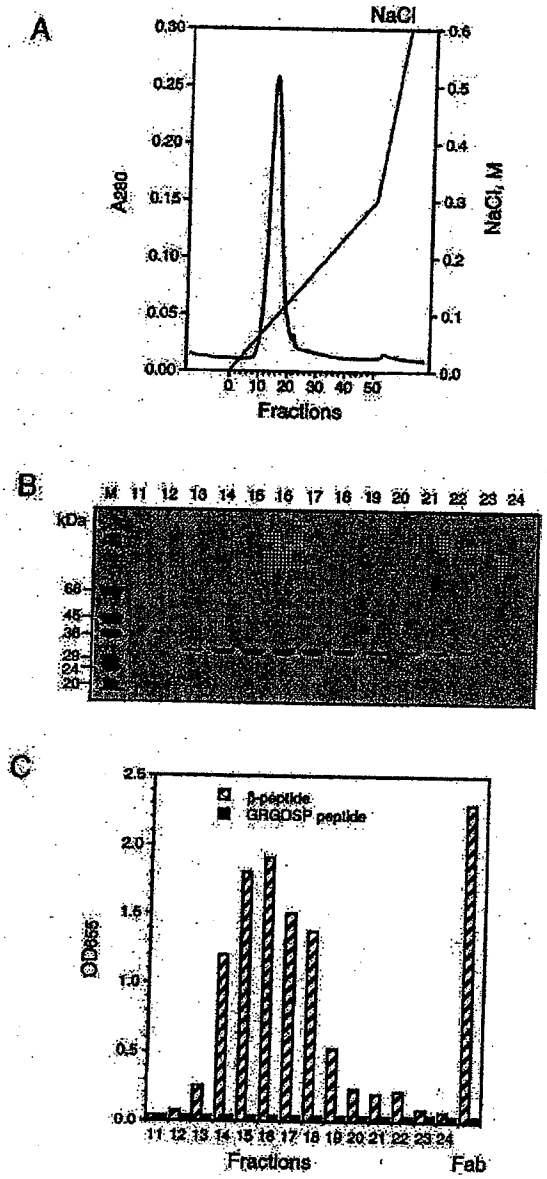

Figure 5. Purification of $scFv_{59D8}$. A, Elution profile of ion-exchange chromatography on MonoS column that followed immobilized metal affinity chromatography. B, Purified single-chain antibody in SDS-PAGE (12%) analysis of chromatographic fractions after staining with Coomassie brilliant blue. C, Binding of eluted fractions (5 μL each) to immobilized β-peptide compared with Fab of original 59D8 antibody (5 μL; 0.3 mg/mL). GRGDSP peptide was used as negative control.

because this substitution has been shown to give increased yields of scFvs.[17] Indeed, the yield of functional soluble $scFv_{59D8}$ was increased ≈4 times by this single amino acid substitution.

The factor Xa recognition sequence and the hirudin sequences were fused to the $scFv_{59D8}$ by PCR. However, only a low yield of soluble fusion protein was obtained with the pHOG21 expression vector. This was probably due to the high cysteine content (10%) of hirudin that might interfere with the folding process of soluble scFv. To obtain higher

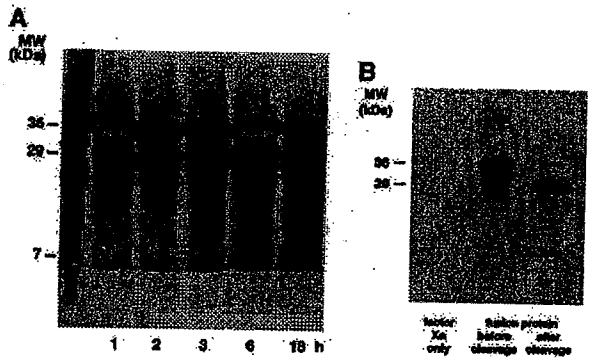

Figure 6. Cleavage of $scFv_{59D8}$-Xa-hirudin by factor Xa. After affinity purification on a β-peptide column, $scFv_{59D8}$-Xa-hirudin was digested by factor Xa, resolved by 12.5% SDS-PAGE, and stained with 0.1% silver nitrate (A) or immunoblotted by anti-c-myc antibody (B). With longer duration of cleavage by factor Xa at room temperature, staining of fusion protein (36 kDa) decreased, whereas staining of both cleavage products, scFv fragment (29 kDa) and hirudin (7 kDa), increased (A). B, After nearly complete cleavage (37°C; 5 hours), immunoblots revealed positive anti-c-myc staining corresponding to fusion protein (36 kDa) and scFv fragment (29 kDa). No staining by anti-c-myc mAb was obtained for factor Xa or hirudin. HRP-conjugated goat anti-mouse Ab was used to visualize anti-c-myc staining. MW indicates molecular weight.

levels of the $scFv_{59D8}$-Xa-hirudin fusion protein, we chose the expression vector pOPE51,[17] which facilitates the production of large amounts of fusion proteins as inclusion bodies in the periplasmic space. When this expression system was used, up to 10 mg of highly purified $scFv_{59D8}$-Xa-hirudin could be obtained from a bacterial culture of 5 L.

The fusion protein $scFv_{59D8}$-Xa-hirudin was analyzed by SDS-PAGE and tested for its binding to β-peptide and its susceptibility to factor Xa cleavage. The molecular weight of the intact fusion protein $scFv_{59D8}$-Xa-hirudin was 36 kDa, that of the cleavage product $scFv_{59D8}$-Xa was 29 kDa, and that of hirudin was 7 kDa (Figure 6).

We evaluated the functional characteristics of $scFv_{59D8}$-Xa-hirudin by measuring its binding to β-peptide and by determining its antithrombin activity after binding to β-peptide. Binding to β-peptide was comparable to the binding of equimolar amounts of the Fab′ fragment of the original mAb 59D8 as measured in ELISA (Figure 7A). The antithrombin activity of the $scFv_{59D8}$-Xa-hirudin was determined in the presence and absence of factor Xa. $ScFv_{59D8}$-Xa-hirudin was allowed to bind to β-peptide, and the nonbound fusion protein was washed away. The binding function and antithrombin activity of bound $scFv_{59D8}$-Xa-hirudin could thus be evaluated simultaneously. The uncleaved $scFv_{59D8}$-Xa-hirudin revealed no antithrombin activity, whereas $scFv_{59D8}$-Xa-hirudin in the presence of factor Xa demonstrated marked antithrombin activity (Figure 7B).

The ability of the fusion protein to inhibit clot growth was tested in a whole blood clot assay. Native hirudin and $scFv_{59D8}$-Xa-hirudin were directly compared for their ability to inhibit appositional clot growth. $ScFv_{59D8}$-Xa-hirudin was able to inhibit clot growth significantly better than native hirudin (Figure 8).

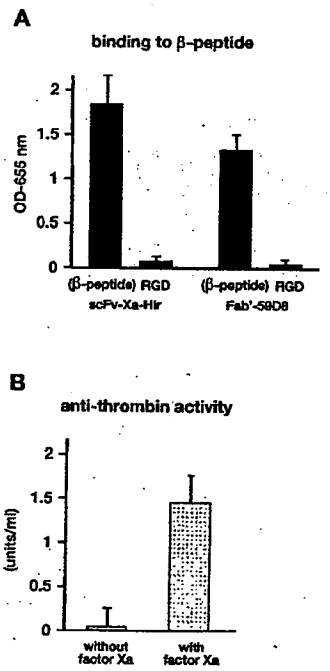

Figure 7. Comparable binding of equimolar amounts of $scFv_{59D8}$-Xa-hirudin and Fab' 59D8 (A) and antithrombin activity of $scFv_{59D8}$-Xa-hirudin bound to β-peptide only after cleavage by factor Xa (B). A, Binding of $scFv_{59D8}$-Xa-hirudin on immobilized β-peptide was detected by mouse anti-c-myc mAb and saturating concentrations of secondary goat HRP-conjugated anti-mouse Ab. Binding of Fab' 59D8 was detected by saturating concentrations of secondary antibody alone. For estimation of unspecific binding, samples were also incubated on immobilized GRGDSP peptide. B, Peptide substrate S-2238 was used to determine thrombin enzymatic activity. Chromogenic reaction was performed in triplicate. Mean values and SDs are shown for representative experiments in relation to standard curve established with native hirudin.

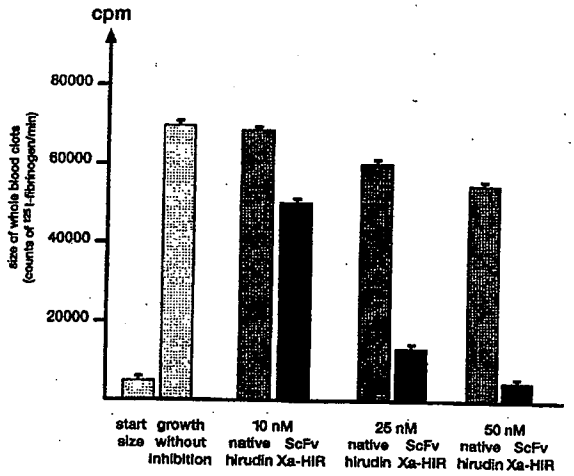

Figure 8. Inhibition of appositional clot growth by native hirudin and $scFv_{59D8}$-Xa-hirudin (ScFv Xa-HIR) in a whole blood clot assay. Clots were created as described in Methods, and size is depicted in counts per minute. Maximal appositional growth is given as growth without inhibition. Inhibition of appositional growth is demonstrated for various concentrations of native hirudin and $scFv_{59D8}$-Xa-hirudin. Mean values and SDs are shown for a representative experiment.

Discussion

Fibrin targeting allows for local enrichment of fibrinolytic agents at the site of the thrombus at low systemic concentrations and thus represents a strategy to increase fibrinolytic potency.[10,11] Furthermore, a chemical conjugate of the fibrin-specific mAb 59D8 and the direct thrombin inhibitor hirudin inhibited fibrin deposition on experimental clots[12] and demonstrated an increase in antithrombotic potency in baboons.[13] To increase the yield and activity of antibody-targeted hirudin and to further improve the risk/benefit ratio of anticoagulation, we have developed a recombinant fusion molecule consisting of an antifibrin single-chain antibody and hirudin. In addition to fibrin targeting, the generation of a free N-terminus, which is essential for the antithrombin activity of hirudin, forms the basis of a unique pharmacological approach. By the addition of the factor Xa recognition sequence, the fusion protein inhibited thrombin only in the presence of factor Xa. Because this factor is part of the activated clotting system and is an important determinant of the procoagulant activity of whole blood clots and arterial thrombi,[15] the designed fusion protein represents an anticoagulant that promises to be preferentially active at the site where it is needed.

The potential therapeutic use of single-chain antibody fusion proteins has several major advantages. The variable regions of antibodies comprise the smallest fragments containing a complete antibody binding site, and fusion molecules can be created without loss in binding function of the scFv. Therefore, scFvs are attractive tools for the targeting of drugs, toxins, and radionuclides. The fusion protein $scFv_{59D8}$-Xa-hirudin with the small molecular size of 36 kDa is expected to be only minimally, if at all, immunogenic, and its small size may improve thrombus accessibility and penetration. It can be produced in bacteria in large amounts, in a short time, and at low cost, and it can be highly purified by affinity chromatography with β-peptide columns, thus providing an ideal situation for drug preparation on a large scale.

Fibrin is an obvious target to concentrate antithrombotic or fibrinolytic agents at the clot. Sufficient amounts of fibrin are present even in platelet-rich thrombi.[13] In addition to mAb 59D8, the mAb MA-15C5, directed against the fragment D-dimer of cross-linked human fibrin, has been used successfully to target plasminogen activators to clots.[19]

Several reports imply that direct thrombin inhibitors may be superior to heparin.[3–8] This could be explained by a number of distinct mechanisms. In contrast to heparin, which only inhibits thrombin as a soluble molecule, hirudin can also inhibit thrombin that is bound to the clot or to soluble fibrin degradation products.[20,21] Heparin binds to various other partners besides thrombin and is thereby inhibited.[22] In contrast to heparin, hirudin has no natural inhibitors.[7] Furthermore, hirudin can displace thrombin from platelet thrombin receptors.[7] In an experimental study, hirudin but not heparin was even able to dissolve preexisting mural thrombi.[7] Nevertheless, the experimental advantages of hirudin compared with heparin have not been reflected by superior clinical performance. Bleeding complications with higher

doses of hirudin appear to be the major limitation.[4] Fusion proteins, such as the one described, provide a promising new development based on the strategy of targeting to and activation at the existing or developing thrombus. This may result in highly efficient inhibition of thrombin and at the same time in fewer bleeding complications.

In summary, a fusion protein has been developed that combines fibrin targeting and antithrombin activity after activation by factor Xa. This recombinant anticoagulant promises to be active only when and where it is needed, thus providing a pharmacological approach that may facilitate an effective anticoagulation without systemic bleeding complications.

Acknowledgments

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